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Spectrally distinct cytochrome *b*-563 components in a chloroplast cytochrome *b–f* complex: Interaction with a hydroxyquinoline *N*-oxide

(photosynthetic electron transport / plastoquinol:plastocyanin oxidoreductase / 2-heptyl-4-hydroxyquinoline *N*-oxide)

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ABSTRACT **The two heme equivalents of cytochrome *b*-563 in the photosynthetic cytochrome *b–f* complex can be distinguished by their rate of reduction with dithionite at 25°C and by their optical absorption spectra at 77 K. The cytochrome *b* component that is rapidly reduced after addition of dithionite or reduced ferredoxin possesses an α band that splits at 77 K into two peaks, at 557 and 561 nm. Prolonged incubation with reductant reveals a second, approximately equimolar cytochrome *b* component that has at 77 K an unsplit α-band maximum at 561 nm. The designations cytochrome *b*-563$_H$ and cytochrome *b*-563$_L$, respectively, are proposed for the rapidly and more slowly reduced cytochrome *b*-563 components. Potentiometric titration establishes a midpoint potential, E_m, of −30 mV (electron change $n \approx 2$) for cytochrome *b*-563$_H$ and −150 mV ($n = 1$) for cytochrome *b*-563$_L$ at pH 7.5. The reduction potential of these components is raised by 2-heptyl-4-hydroxyquinoline *N*-oxide, giving E_m values of +57 and −34 mV, respectively, with each titration slope approximating $n = 2$.**

The ubiquinol:ferricytochrome *c* oxidoreductases (EC 1.10.2.2) of mammalian and yeast mitochondria (1, 2) and of photosynthetic bacteria (3) are characteristically comprised of an iron–sulfur center, cytochrome c_1, and two cytochromes *b*. Detailed studies of such cytochrome *b*–c_1 complexes have been greatly facilitated by the ability to distinguish by spectrophotometry and potentiometry between the constituent *b*-type cytochromes [cytochromes *b*-562 and *b*-566, with $E_m \approx +40$ and −100 mV, respectively, in the case of bovine heart complex III (2); E_m is the midpoint potential]. The plastoquinol:plastocyanin oxidoreductase of chloroplasts contains an iron–sulfur center, the *c*-type cytochrome *f*, and two equivalents of cytochrome *b*-563 (cytochrome b_6) (4). These cytochrome *b* heme equivalents, however, have not been distinguished by spectrophotometry at room temperature. Attempts to differentiate between them by redox potentiometry have given equivocal results owing to their apparent lability (4, 5); for example, biphasic reductions of cytochrome *b*-563 were reported in chloroplasts treated with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (6) and in an isolated, four-component cytochrome *b–f* complex (7), whereas monophasic reductions were reported for native chloroplasts (6, 8). Freeze-quenching during reductive titration (9) has provided evidence for spectral heterogeneity of cytochrome *b*-563 in the isolated complex. [The report of Hurt and Hauska (9), which appeared during the final preparation of this report, complements part of the work presented here.]

A potent inhibitor of respiration, antimycin A, binds strongly to *b*–c_1 complexes. It perturbs cytochromes *b*-562 and *b*-566, shifting their E_m values to higher potentials and inducing a wavelength shift in the absorption maximum of cytochrome *b*-562 (1, 2). Antimycin A binds relatively weakly to thylakoids (10) and probably inhibits turnover of the cytochrome *b–f* complex only when this is dependent on cyclic flow of electrons around photosystem I (10, 11). The quinone analog 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) resembles antimycin A in its effect on mitochondria (2, 12), and it is, in addition, an effective inhibitor of linear electron transport in chloroplasts (13, 14).

In the study reported here, we have used freeze-quenching and low-temperature (77 K) spectrophotometry to establish the presence of two distinct cytochrome *b*-563 components in a five-component cytochrome *b–f* complex (15) isolated from spinach. These components differ in E_m at 20°C, and both are sensitive to HQNO, which raises their reduction potentials and alters the apparent electron change for the lower potential component from $n = 1$ to $n = 2$.

MATERIALS AND METHODS

Cytochrome *b–f* complex was isolated from spinach chloroplasts by a procedure derived from that of Hurt and Hauska (7), which is described in detail elsewhere (15). Low-temperature spectra were obtained with a vertical-optics spectrophotometer of our own design. The cuvettes were 25 mm in diameter and gave an optical depth of 2–3 mm; samples were frozen by immersion of the cuvettes in liquid N_2 and were not devitrified. Room-temperature spectra were obtained with a dual-wavelength spectrophotometer (16). Spectral data were stored and manipulated digitally on a PDP 11/34 computer interfaced with the spectrophotometers.

Potentiometric titrations were performed in 20 mM sodium *N*-tris(hydroxymethyl)methylglycine (Tricine)/0.5% cholate/100 mM KCl, pH 7.5, containing 10 μM of each of the following redox mediators: dichloroindophenol (E_m at pH 7 = +217 mV), 5-hydroxy-1,4-naphthoquinone (+30 mV), duroquinone (+5 mV), 2-methyl-1,4-naphthoquinone (−10 mV), and 2-hydroxy-1,4-naphthoquinone (−145 mV). Dimethyl sulfoxide was used as the solvent for mediators other than dichloroindophenol and so was present at 0.4% during titrations. These were carried out under argon (17) by addition of dithionite dissolved in Na_2CO_3 buffer. Ambient potential was monitored by using a Pt indicator electrode with a calomel reference (1.0 M KCl) electrode. The accuracy of the potentiometric system was checked against the ferricyanide/ferrocyanide couple (18). Spectral scans between 540 and 580 nm were recorded for each point, though no sig-

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Abbreviations: HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; Tricine, *N*-tris(hydroxymethyl)methylglycine; E_m, midpoint potential.

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nificant shifts of baseline or peak wavelength were observed. Menadiol was prepared by reduction with dithionite (19). Ferredoxin was isolated as described (20). An extinction coefficient of 19 $mM^{-1}cm^{-1}$ at 608 nm was used for indigo-5,5'-disulfonate.

RESULTS

The cytochrome *b–f* complex used here (15), like that isolated by Hurt and Hauska (7, 9, 19), contains only one class of *b*-heme cytochrome: cytochrome *b*-563 (b_6). No cytochrome *b*-559 is detectable by reduction with menadiol (9, 19). Cytochrome *f* is the only cytochrome component of the complex that is reduced by addition of hydroquinone or ascorbate.

Electrochemical properties of the cytochrome *b–f* complex were explored by potentiometric titration. Cytochrome *b*-563 was reduced in two steps with midpoints at −20 mV and −153 mV (Fig. 1) at pH 7.5. The higher potential transition was characteristically sharp, with a slope corresponding to $n = 2$ (17), whereas the second step of reduction had a slope of approximately $n = 1$. In replicate titrations, the midpoints obtained were −30 ± 10 mV and −150 ± 15 mV (range) for titrations carried out over 1–3 hr. Inclusion of additional mediators (*N*-methylphenazonium methosulfate, toluylene blue, benzoquinone, or 9,10-anthraquinone-1,5-disulfonate) did not significantly alter these values, and nearly identical reduction profiles were obtained for samples retitrated after back-oxidation in air (data not shown).

Inclusion of 20 μM HQNO in the medium dramatically altered the reductive titration curve. The midpoint potentials were raised to +57 and −34 mV (Fig. 1), each with a slope near that expected for an $n = 2$ transition. Nonlinear least-squares curve-fitting of the data to independent Nernst equations (17) apportioned 57 ± 2% and 59 ± 2% (SEM) of the cytochrome *b*, with and without HQNO respectively, to the first reduction step (note that all data points contribute to these estimates). This

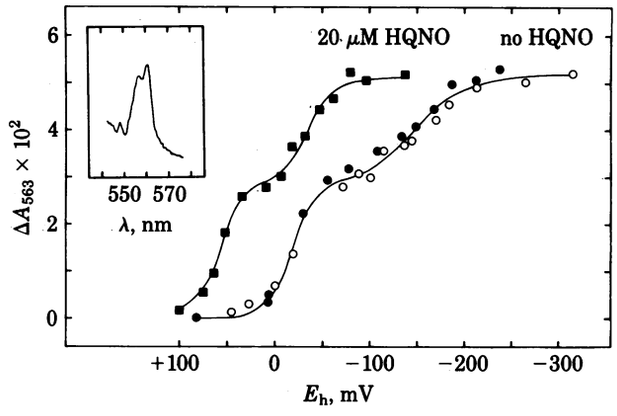

FIG. 1. Effect of HQNO on the reductive titration of cytochrome *b*-563 in the cytochrome *b–f* complex at 20°C. Complex (1.6 μM in cytochrome *f*) was titrated with sodium dithionite in the presence (■) or absence (●, ○) of 20 μM HQNO. Each line shown is the least-squares best fit to two independent Nernstian reductions with $n = 2$ for all transitions except the one at low potential without HQNO, for which $n = 1$ was assumed. Reduction of cytochrome *b*-563 was evaluated from the absorbance change $\Delta A_{563-575}$ after equilibration. The buffer was 20 mM sodium Tricine, pH 7.5/0.1 M KCl/0.5% cholate with mediators as described. Ambient potential, E_h, is referred to the standard hydrogen electrode. (*Inset*) Absorption spectrum at 77 K obtained by addition (prior to freezing) of 1 mM menadiol to the cytochrome *b–f* complex (3 μM cytochrome *f*) in the presence of 40 μM HQNO minus the spectrum obtained in the absence of HQNO. The buffer was 20 mM sodium Tricine/0.5% cholate/20% glycerol, pH 7.5, containing 400 units of catalase per ml and 1 mM hydroquinone.

constant proportion of higher-potential cytochrome *b*-563 heme to lower-potential heme supports the notion that HQNO shifts the entire redox titration profile to more positive values (the alternative interpretation being that the −150-mV transition seen without HQNO switches to +57 mV with HQNO). Addition to the cytochrome *b–f* complex of hydroquinone (to reduce cytochrome *f*), followed by menadiol in the presence or absence of HQNO, yielded the low-temperature difference spectrum shown in Fig. 1 *Inset*. Comparison with the difference spectrum for reduction of cytochrome *f*, as an internal standard, showed that 15% of the cytochrome *b*-563 is reduced under these conditions. This fraction clearly has a split α band at 77 K with maxima at 557 and 561 nm.

The spectral characteristics of cytochrome *b*-563 also were examined by freezing aliquots of cytochrome *b–f* complex in liquid N_2 at increasing intervals after addition of a few grains of dithionite (Fig. 2); the first time point is determined by the 20 s required for sodium dithionite to dissolve. These spectra of cytochrome *b*-563 include two distinct components: one is rapidly reduced by dithionite and possesses a split α band with maxima as 557 and 561 nm; the second equivalent of cytochrome *b* becomes reduced only after much longer incubation with dithionite. Subtraction of spectra taken at short and long times after dithionite addition (Fig. 2*B*) revealed that the slowly reduced cytochrome *b* had no absorption peak at 557 nm. Thus, the shoulder observed when the full cytochrome *b* heme complement is reduced must arise principally from the 557-nm absorption peak of the rapidly reduced component.

Because the spectral envelope of the rapidly reduced cytochrome is identical to that obtained for high-potential cytochrome *b*-563 by partial reduction with menadiol in the presence of HQNO (Fig. 1 *Inset*), we designate this component cytochrome b-563_H. This seems preferable to subscripts "HP" and "LP" (9), owing to historical association of these with forms of chloroplast cytochrome *b*-559 that are not well characterized (4) and are unlikely to be interrelated in an analogous fashion to the cytochrome *b*-563 components described here. The more

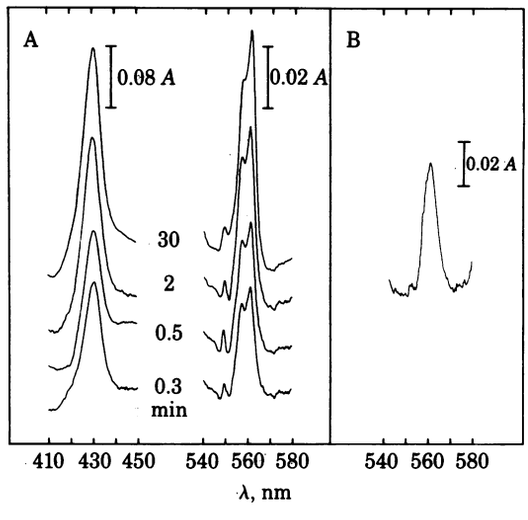

FIG. 2. Time course of cytochrome *b*-563 reduction. (*A*) Ascorbate-reduced cytochrome *b–f* complex (1.6 μM cytochrome *f*) in 20 mM sodium Tricine/0.5% cholate/20% glycerol, pH 7.5, was frozen 20 s, 30 s, 2 min, or 30 min after addition of a few grains of solid dithionite. For the 2-min spectrum, the cuvette was purged with N_2 during reduction. For the 30-min spectrum, the sample was reduced in a sealed vial and transferred to the N_2-purged cuvette immediately before freezing. The spectrum for ascorbate-reduced complex was subtracted from all spectra shown. Other conditions were as for Fig. 1 *Inset*. (*B*) Difference between spectra taken 20 min and 30 s after addition of dithionite.

slowly reduced component, designated cytochrome b-563$_L$, corresponds to the low-potential transition, as shown below.

The cytochrome γ band showed no splitting or shift at any stage of the reduction, indicating that only cytochrome b-563 is involved (21, 22). Comparable results were obtained using the cytochrome b–f complex isolated by the procedure of Hurt and Hauska (7).

Two distinct cytochrome b-563 components also were evident (Fig. 3A) in the course of reduction of the cytochrome b–f complex by ferredoxin (23). This time series clearly showed that the initially reduced cytochrome b-563$_H$ has an asymmetric absorption band even at 20°C, whereas the fully reduced cytochrome b-563 complement is more symmetric. A short-wavelength isosbestic point was observed at 548 nm throughout the course of reduction, whereas the long-wavelength crossover point shifted from 570.5 nm (presumably for cytochrome b-563$_H$) to 572.0 nm (for cytochrome b-563$_L$). A slight red shift in absorbance maximum also was observed during reduction. The spectrum at 77 K for fully reduced cytochrome b-563 with dithionite (Fig. 2) as reductant was essentially identical to that obtained by using ferredoxin (Fig. 3B) as reductant.

Partial back-oxidation of the ferredoxin-reduced complex with indigo-5,5′-disulfonate allowed direct visualization of cytochrome b-563$_L$ at equilibrium (Fig. 3). The difference spectrum for fully reduced cytochrome b minus partially reoxidized cytochrome b (i.e., cytochrome b-563$_L$) was sharp and symmetrical both at 20°C (Fig. 3A *Inset*) and at 77 K (Fig. 3B *Inset*). In Fig. 3A *Inset*, 13% of the total cytochrome b was oxidized in equilibrium with 8% oxidized indigo-5,5′-disulfonate (E_m = −146 mV at pH 7.5; ref. 24), whereas 24% oxidation of cytochrome b was observed in equilibrium with 40% oxidation of this dye (Fig. 3B *Inset*). These values indicate an n = 1 redox transition centered at −154 mV for 46% of the cytochrome b-563, consistent with values obtained by reductive titration in the presence of quinone mediators (Fig. 1).

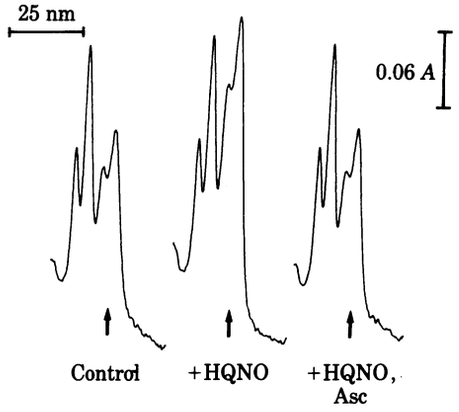

FIG. 4. Effects of HQNO on the reduction of cytochrome b–563 by dithionite. Low-temperature (77 K) spectra shown are of cytochrome b–f complex (3 μM cytochrome f) frozen 40 s after addition of dithionite. Samples also contained 40 μM HQNO or 1 mM ascorbate (Asc), as indicated, before addition of dithionite. The arrows indicate 560 nm. Buffers were as described in Fig. 2.

The reduction of cytochrome b-563$_L$ by dithionite is greatly accelerated by HQNO (Fig. 4), which enhances flash-induced cytochrome b-563 reduction in thylakoids (13, 14). The potentiation of cytochrome b-563$_L$ reduction by HQNO is lessened by preincubation with ascorbate (Fig. 3), probably owing to re-

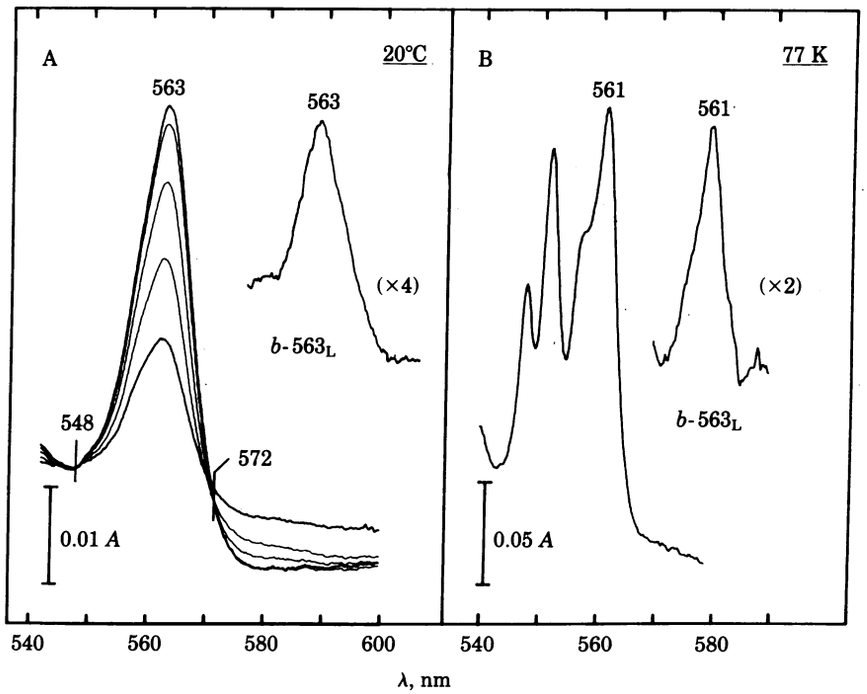

FIG. 3. Reduction of cytochrome b-563 by ferredoxin. Cytochrome complex (1.3 μM cytochrome f) was reduced by addition of 300 μM NADPH to an anaerobic medium (23) containing 1.6 μM spinach ferredoxin, 0.1 unit of ferredoxin:NADP reductase per ml, and 20 μM dinitrophenyl ether of iodonitrothymol in 40 mM sodium Tricine/0.5% cholate, pH 7.5/20% glycerol/0.4% ethanol. (*A*) Spectra at 20°C were recorded 2, 16, 32, 53, 80, and 120 min after NADPH was added, with 545 nm as the reference wavelength. The spectrum obtained before addition of NADPH was digitally subtracted from each scan shown. (*Inset*) The difference spectrum for fully reduced complex (120-min record) versus complex partially reoxidized by addition of indigo-5,5′-disulfonate to 304 μM. The spectrum was digitally corrected for absorbance of the oxidized indigo-5,5′-disulfonate present (24 μM). (*B*) Absorption spectrum at 77 K for cytochrome b–f complex frozen after full reduction with ferredoxin; the spectrum of reduced cytochrome f, with peaks at 548 and 542 nm, has not been subtracted. (*Inset*) Difference spectrum at 77 K for fully reduced cytochrome b–f complex minus complex reoxidized with 390 μM indigo-5,5′-disulfonate [corrected for absorbance of the oxidized indigo-5,5′-disulfonate present (156 μM)].

duction of the nitroxide group (25). These observations are in accord with the positive shift in E_m induced by HQNO in both cytochrome *b*-563 components (Fig. 1). The dinitrophenyl ether of iodonitrothymol, which interacts with the cytochrome *b–f* complex at the Rieske iron–sulfur center, accelerated reduction of cytochrome *b*-563 by ferredoxin (23) but did not affect reduction by dithionite (data not shown).

DISCUSSION

Absorption maxima at 557 nm in the low-temperature spectra of thylakoids and chloroplast extracts generally have been attributed to cytochrome *b*-559 (4, 8, 26). Stuart and Wasserman (27) reported maxima at 557 and 561 nm (at 77 K) for an isolated, reduced cytochrome *b*-563; other workers suggested, however, that this α-band splitting is an artefact of purification (5). Cox used the difference spectra between prolonged and brief incubation with dithionite to obtain a low-temperature spectrum of cytochrome *b*-563 having a shoulder but no secondary peak at 557 nm (26).

The procedure employed here to isolate the cytochrome *b–f* complex avoids the use of Triton X-100 and is mild enough to preserve plastoquinol:plastocyanin oxidoreductase activity (15). Furthermore, this preparation contains no cytochrome *b*-559. Nevertheless, spectra of the cytochrome *b–f* complex frozen in the initial phase of reduction by dithionite display two α-band absorption peaks, at 557 and 561 nm (Fig. 2). The same envelope was observed for complex reduced with menadiol in the presence of HQNO (Fig. 1 *Inset*), even though the absorbance obtained was only ≈30% of that observed 30 s after adding dithionite, or ≈15% of the total cytochrome *b* absorbance. Hence, a single species probably gives rise to the 557- and initial 561-nm peaks as suggested by Hurt and Hauska (9) and as supported (22) by the absence of any splitting in the γ-band absorption maximum (Fig. 2).

The 561-nm peak of reduced cytochrome *b*-563_L is not due to a collapse of the 557- and 561-nm peaks of cytochrome *b*-563_H into a single envelope, as might possibly result from denaturation during long incubation with dithionite (28). Such a spectral shift would lead to regions of negative absorbance in difference spectra between sequential time points within 30 s and 20 min of dithionite addition, whereas no bleaching is seen. Furthermore, reduction by ferredoxin in the presence of ferredoxin:NADP reductase, NADPH, and the dinitrophenyl ether of iodonitrothymol gave essentially the same spectrum as did extended reduction by dithionite (Fig. 3). The split and unsplit difference spectra at 77 K also can be obtained by partial reduction and partial reoxidation of the cytochrome *b–f* complex (Fig. 1 *Inset* and Fig. 3*B Inset*, respectively), indicating that only two spectral species are necessary to account for the changes observed.

Hurt and Hauska were first to report (7) that the broad curve obtained upon potentiometric titration of cytochrome *b* in isolated cytochrome *b–f* complex could be resolved arithmetically into two one-electron transitions with E_ms of −60 mV and −180 mV (pH 7.4). In our experience, approximately half of the cytochrome *b* becomes reduced at about −30 mV in a sharp transition, the remainder exhibiting a broad reduction profile (Fig. 1) centered at −150 mV. These different results may be attributable to differences in pH, ionic strength, and detergent concentrations used in the respective titrations or to differences in the isolation procedure. A significant factor also may be the lability of the cytochrome *b–f* complex to ferricyanide, which irreversibly inhibits oxidoreductase activity (data not shown). In the present work, the complex was not subjected to oxidation with ferricyanide before reductive titration with dithionite.

The inhibitor HQNO accelerates the second phase of cytochrome *b*-563 reduction by dithionite (Fig. 4), evidently by shifting the apparent reduction potentials of both components to higher potential (Fig. 1). This effect on the E_m values may be attributable to the structural homology between HQNO and the semiquinone anion of plastoquinone; binding at one or more plastoquinone binding sites near the cytochrome *b* hemes in the *b–f* complex may stabilize the reduced cytochrome *b* hemes, that is, raise their E_ms. This would lead to the observed inhibitions by HQNO of cytochrome *b*-563 (b_6) reoxidation (13, 14) and of the slow phase of the electrogenic reaction (14).

HQNO also elicits sharp reductive transitions for both steps. The $n = 2$ slopes observed do not necessarily indicate two-electron reductions of cytochrome, although this might indeed be so for a dimeric complex containing four equivalents of cytochrome *b*. Transitions may be affected by cooperative interactions between the cytochrome *b–f* complex and mediator quinones (17), particularly if the complex carries sites that stabilize semiquinones (29). Redox transitions involving interaction with noncovalently bound plastoquinone (19) or HQNO could be expected to affect the course of cytochrome *b*-563 reduction. Conformational or electronic interactions between the *b* hemes within the complex also may modify potentiometric behavior.

Slow equilibration, too, can distort redox transitions. The insensitivity of the redox titration profile to additional mediators or to variations in the rate of titration argue against this possibility (17), as does the reversibility of cytochrome *b*-563_L reduction by indigo-5,5′-disulfonate. Oxidative titration is not a satisfactory control in the case of the isolated complex (see also refs. 7, 9, and 19), owing to its sensitivity to ferricyanide, already noted. Furthermore, stability of HQNO in the presence of reductants is questionable; irreversible reduction of the nitroxide group may occur at low potential (30).

It generally has been assumed that chloroplast cytochrome *b*-563 is a single, homogenous species. The many analogies now recognized between the cytochrome *b–f* and cytochrome *b–*c_1 complexes (4) and the work of Hurt and Hauska (7) suggest that the well-defined cytochrome *b* heterogeneity of mitochondrial complex III extends to the corresponding complex in the thylakoid membrane. In the latter instance, though, the α-band absorption maxima of the two reduced cytochrome *b* components cannot be resolved at room temperature. We propose that these two components of cytochrome *b*-563 be designated cytochrome *b*-563_H and cytochrome *b*-563_L, where subscripts "H" and "L" refer to high and low potential, respectively. Reduced cytochrome *b*-563_H possesses a double α band at 77 K, while cytochrome *b*-563_L has an asymmetric but solitary peak.

It is important to note that in studies of less well-resolved systems, a 557-nm absorption peak at 77 K will include contributions from both reduced cytochrome *b*-559 and cytochrome *b*-563_H. A recent report by Peters *et al.* (31) illustrates this point well. They combined potentiometric titration with low-temperature spectroscopy and spectral deconvolution to estimate the cytochrome contents of photosystem 1 particles extracted with digitonin. Making the assumption that cytochrome *b*-563 is spectrally homogenous, they found a cytochrome *b*-563/*b*-559_{LP}/*f* ratio of ≈1.5:2.5:1.0. Reassessment in the light of our findings suggests that their data are also consistent with a ratio of 2.0:1.5:1.0, with cytochrome *b*-563_H having an E_m near −50 mV and peaks at both 557 and 561 nm. This ratio is more in line with literature values obtained using redox buffers (8, 32).

If a Q-cycle scheme is applicable to both the cytochrome *b–*c_1 and *b–f* complexes (4), cytochrome *b*-563_L should correspond

to cytochrome *b*-566. This would place it closest to the Rieske iron–sulfur center, where it would accept an electron from a locally generated plastosemiquinone. The oxidation of cytochrome b-563_L by cytochrome b-563_H (analogous to cytochrome *b*-562 in complex III and cytochrome *b*-561 in *Rhodopseudomonas sphaeroides*) would then follow. Recent evidence indicates that the slow electrogenic step in *R. sphaeroides* is associated with the antimycin-sensitive reoxidation of cytochrome *b*-561 by quinone (33). HQNO inhibits the rate of cytochrome b_6 reoxidation in thylakoids (13) and so may inhibit the slow electrogenic step (14) in a comparable fashion. Much more work is required to confirm and amplify these observations and to develop a general understanding of the role of the low potential pathway involving cytochrome *b* hemes within the framework of a Q-cycle or other mechanism.

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